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(54) Title: PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES MADE THEREFROM

(57) Abstract

Mutants of pneumolysin that are non-toxic by reason of amino acid substitutions have been constructed. These mutants elicit an immune response in animals that is reactive to wild-type pneumolysin. The invention also encompasses vaccines for humans based on these mutants, including vaccines comprising conjugates with pneumococcal capsular polysaccharides.

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PNEUMOLYSIN MUTANTS AND PNEUMOCOCCAL VACCINES MADE THEREFROM

This invention relates to mutants of the toxin pneumolysin and pneumococcal vaccines based on these mutants.

BACKGROUND

Streptococcus pneumoniae (pneumococcus) is an important pathogen, causing invasive diseases such as pneumonia, meningitis and bacteraemia. Even in regions where effective antibiotic therapy is freely available, the mortality rate from pneumococcal pneumonia can be as high as 19% in hospitalized patients and this increases to 30-40% in patients with bacteraemia. These high mortality rates have been reported in the U.S.A. where pneumonia, of which *S. pneumoniae* is the commonest cause, is the fifth ranking cause of death. Indeed, pneumonia is the only infectious disease amongst the top ten causes of death in that country. In the United States mortality rates for pneumococcal meningitis range from 13-45%. In developing countries, in excess of 3 million children under the age of 5 years die each year from pneumonia, and again *S. pneumoniae* is the commonest causative agent. *S. pneumoniae* also causes less serious, but highly prevalent infections such as otitis media and sinusitis, which have a significant impact on health-care costs in developed countries. Otitis media is especially important in young children; sinusitis affects both children and adults.

In the late 1970's, a vaccine was licensed for the purpose of preventing serious infections, especially bacterial pneumonia and for protecting certain groups, such as splenectomized individuals and young children, who are particularly susceptible to fulminating pneumococcal disease. The vaccine is composed of purified capsular polysaccharides, which are the predominant pneumococcal surface antigens. However, each serotype of *S. pneumoniae* (of which there are 83) has a structurally distinct capsular polysaccharide, and immunization with one serotype confers no protection whatsoever against the vast majority of the others. The vaccine currently licensed in Australia contains polysaccharides purified from the 23 most common serotypes, which account for approximately 90% of pneumococcal infections in this country.

Protection even against those serotypes contained in the vaccine is by no means complete, and there have been several reports of serious, even fatal infections occurring in vaccinated high-risk individuals. The efficacy of the vaccine is poorest in young children, and several studies, including one conducted in Adelaide, have shown that the existing formulation has little or no demonstrable clinical benefit in this group. This apparent failure of the vaccine appears to be related to the poor immunogenicity of certain pneumococcal polysaccharides in children under 5 years of age. We have shown that the antibody response is particularly poor to the five serotypes which most commonly cause disease in children (types 6, 14, 18, 19 and 23). Indeed, the antibody response to these pneumococcal polysaccharides only approaches adult levels in children over 8 years of age at the time of vaccination.

In view of this, a vaccine, including antigens other than the capsular polysaccharides seems to be required to protect young children from pneumococcal infection. One such antigen could be pneumolysin, a protein toxin produced by all virulent *S. pneumoniae* isolates. Immunization of mice with this protein has been found to confer a degree of protection from pneumococcal infection.

However there is a difficulty in that pneumolysin is toxic to humans. Thus pneumolysin included in a vaccine must therefore be substantially non-toxic. However, the rendering of a pneumolysin non-toxic by most currently employed methods would be likely to alter the basic configuration of the protein so as to be immunogenically distinct from the native or wild-type pneumolysin. An immune response elicited by an altered protein that is immunogenically distinct from the native pneumolysin will have a decreased protective capacity or no protective capacity. Thus the difficulty is to produce an altered pneumolysin that is non-toxic and at the same time sufficiently immunogenically similar to the toxic form to elicit a protective immune response.

An altered pneumolysin with the above characteristics can then be used in a number of ways in a vaccine. Thus the altered pneumolysin may be used by itself to immunise, or alternatively the altered pneumolysin may be conjugated to pneumococcal polysaccharide, or

alternatively may be included in a vaccine wherein pneumococcal polysaccharides may be conjugated to another protein and the altered pneumolysin is present in a non-conjugated form only. Alternatively, pneumococcal polysaccharide and pneumolysin may both be used in an unconjugated form.

DESCRIPTION OF INVENTION

In a broad form therefore the invention may be said to reside in an altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the altered pneumolysin has reduced complement binding activity as compared to wild-type pneumolysin. Reduction in the complement binding activity results in less inflammation at the site of administering the vaccine.

Preferably the altered pneumolysin has reduced Fc binding activity as compared to wild-type pneumolysin. Reduction in the Fc binding activity results in less inflamation at the site of administering the vaccine.

Preferably the altered pneumolysin is altered by reason of one or more amino acid substitutions relative to wild-type pneumolysin.

The pneumolysin may be altered in that the amino acid present at any one or more than one of residue sites 367, 384, 385, 428, 433 or 435 of wild-type pneumolysin are replaced, removed or blocked.

In a further form the invention could be said to reside in a vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

Preferably the vaccine comprises capsular polysaccharide material conjugated with the altered pneumolysin.

The capsular material may be derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

In this embodiment serotypes which are commonly associated with disease in children, and to which children generally have a poor immune response, may be specifically targeted (i.e. Danish serotypes 6A, 6B, 14, 18C, 19A, 19F and 23F). Other common serotypes contained in the present 23-valent Merck Sharp and Dohme vaccine (Pneumovax 23) however, could also be used to synthesize conjugates (i.e. types 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F) or indeed any other serotype. Conjugation of any pneumococcal polysaccharides to the protein carrier ensures good T-cell dependent immunogenicity in children, such that protective levels of anti-polysaccharide antibody are produced.

The combination of the altered pneumolysin together with the capsular material will ensure an extra degree of protection, particularly against serotypes of *S. pneumoniae* whose polysaccharides are not incorporated in the existing vaccine formulations.

The vaccine is preferably administered by sub-cutaneous injection, with or without an approved adjuvant, such as alumina gel.

In another form the invention could be said to reside in a recombinant clone including a replicon and a DNA sequence encoding an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

In yet another form the invention could be said to reside in a method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from an expression system including a recombinant clone with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal reactive to wild-type pneumolysin.

Preferrably the expression system is a culture of a host cell including a recombinant clone with DNA encoding the altered pneumolysin.

In another form the invention could be said to reside in a method of producing a vaccine including the step of amplifying a recombinant clone encoding an altered pneumolysin, inducing transcription and translation of said cloned material, the purification of altered pneumolysin, and the step of conjugating the altered pneumolysin with a capsular polysaccharide, the altered pneumolysin having substantially reduced toxic activity as compared with wild-type pneumolysin.

For a better understanding of the invention specific embodiments of the invention will now be described with reference to diagrams wherein:-

FIG. 1 Is the DNA sequence of the gene encoding wild-type pneumolysin,

FIG. 2 Is the DNA sequence of an altered gene encoding wild type pneumoltsin used for cloning the pneumolysin gene into an expression vector,

FIG. 3 Is the amino acid sequence of the wild-type pneumolysin as derived from the DNA sequence of the gene encoding the wild type pneumolysin, and

FIG. 4 shows the amino acid sequence of pneumolysin showing amino acid substitutions introduced by site directed mutagenesis.

Recombinant DNA techniques have been used to construct non-toxic pneumolysin derivatives suitable for administration to humans. To achieve this, the *S. pneumoniae* gene encoding pneumolysin was cloned into *Escherichia coli* and its complete DNA sequence determined. The DNA sequence is shown in Figure 1 and the derived amino acid sequence is shown in Figure 3.

Three regions of the pneumolysin gene were subjected to oligonucleotide-directed mutagenesis. The first region encodes amino acids 427 - 437 in the protein sequence, and is indicated by an underline in Figure 3. This 11 amino acid sequence shows absolute homology with similar regions in other related thiol activeted toxins thus is thought to be responsible for the haemolytic acitivity and hence toxic activity of the toxin. The other two regions encode amino acids 257 - 297 and amino acids 368 - 397 and are also indicated by an underline in Figure 3. These two regions of the toxin have substantial amino acid sequence homology with human C-reactive protein (CRP), and by inference therefore, are thought to be responsible for the ability of pneumolysin to bind the Fc region of immunoglobulins and to activate complement. Fifteen separate mutations in the pneumolysin gene, resulting in single amino acid substitutions, were constructed, as shown in Figure 4. In an effort to maintain the structure of the altered pneumolysin, conservative substitutions were made, so that amino acids are substituted with amino acids of a similar nature.

For the region involved in haemolytic activity, Cys_{428} -> Gly, Cys_{428} -> Ser, Trp_{433} -> Phe, Glu_{434} -> Asp and Trp_{435} -> Phe each reduced haemolytic activity by 97%, 90%, 99%, 75% and 90% respectively. The other mutations in that region (Cys_{428} -> Ala, Glu_{434} -> Gln and Trp_{436} ->Phe) did not affect haemolytic activity. Mutating a separate region of the toxin thought to be responsible for binding to target cell membranes also affects haemolytic activity of the protein. This substitution, His_{367} -> Arg, completely inhibits haemolytic activity. This is a quite unpredictable finding in that His_{367} -> Arg therefore shows a greater inhibition of this property than the substitutions made within the 11 amino acid region thought to be responsible for haemolytic activity.

Mutations in the CRP-like domains were tested for ability to activate complement. For Trp_{379} -> Phe, Tyr_{384}-> Phe, Asp_{385} -> Asn, and Trp_{397} -> Phe, complement activation was reduced by 20%, 70%, 100% and 15%, respectively. The other mutations in the CRP-like domains shown in Figure 4 do not reduce complement activation.

Importantly, the above mutations which affect either haemolytic activity or complement activation do not impair the immunogenicity of the proteins, compared with native or wild-type pneumolysin.

Thus although His_{367} -> Arg is the preferred mutation to reduce the haemolytic activity, a combination of two or more mutants effecting reduced haemolytic activity can also achieve a very high level of reduction in haemolytic activity. Similarly Asp_{385} -> Asn is the prefered mutation to achieve reduced complement activation, however a combination of two or more other mutants that reduce the activity to a lesser degree can also be used.

In a preferred embodiment the pneumolysin derivative for use in the vaccine would contain a combination of certain of the above mutations such that the protein is unable to activate complement in addition to having zero haemolytic activity. Examples of such combination are:-

1) His_{367} -> Arg + Asp_{385}-> Asn,
2) His_{367} -> Arg + Asp_{385} -> Asn + either Cys_{428} -> Gly or Trp_{433} -> Phe
3) Asp_{385} -> Asn + Cys_{428} -> Gly + Trp_{433} -> Phe

These then are some preferred combinations, however it is to be understood that other combinations of mutations can be used to make up the altered pneumolysin for use in a vaccine. Further the altered pneumolysin may comprise any one of the individual mutations with sufficiently reduced activity.

High level expression of the altered pneumolysin from DNA encoding the altered pneumolysin can be achieved by using any one of a number of conventional techniques including the expression in a prokaryotic host with the DNA cloned appropriately within any one of the many expression vectors currently available, or cloned appropriately within the host chromosome; expression in a eukaryotic host with the DNA cloned appropriately either within an expression vector or cloned within the host chromosome; or within an *in vitro* expression system such as may comprise purified components necessary for expression of altered pneumolysin.

To achieve high level expression of the mutated pneumolysin gene, it has been cloned into the vector pKK233-2 for expression within *Escherichia coli* or other like prokaryote. This vector included ampicillin and tetracycline resistance genes, the *trc* promoter (which can be regulated by IPTG [isopropyl-ß-D-thiogalactopyranoside]), and a *lac Z* ribosome binding site adjacent to an ATG initiation codon incorporating an *Nco*I restriction site. Immediately downstream from the initiation codon there are restriction sites for *Pst*I and *Hin*dIII, followed by a strong T_1 T_2 transcription terminator. Prior to insertion into pKK233-2, a *Nco*I restriction site was constructed at the 5' end of the pneumolysin coding sequence (at the initiation codon) by oligonucleotide-directed mutagenesis, as shown in Figure 2. This enabled the proximal end of the altered pneumolysin gene to be cloned into the *Nco*I site of pKK233-2; a *Hin*dIII site approximately 80 bases downstream from the pneumolysin termination codon was used to splice the distal end of the altered gene into the compatible site in pKK233-2. The mutant pneumolysin derivative could however, be cloned into any one of a number of high expression vector systems.

The mutant pneumolysin is prepared as follows: *E. coli* cells harbouring the above recombinant plasmid are first grown in 9 litre cultures in Luria Bertani (or any other appropriate) medium, supplemented with the appropriate antibiotic, at 37° C, with aeration. When the culture reaches the late logarithmic phase of growth, IPTG is added to a final concentration of 20µM (to induce expression of the altered pneumolysin gene) and incubation is continued for a further 2 to 3 hours.

Cells are then harvested by centrifugation or ultrafiltration and lysed by treatment with EDTA and lysozyme, followed by sonication, or by disruption in a French pressure cell. Cell debris is removed by centrifugation and the extract is then dialysed extensively against 10mM sodium phosphate (pH7.0). The material is then loaded onto a column of DEAE-cellulose and eluted with a linear gradient of 10-250mM sodium phosphate (pH7.0). Fractions containing peak levels of the pneumolysin derivative are pooled, concentrated by ultrafiltration and loaded onto a column of Sephacryl S-200. This column is developed in 50mM sodium phosphate (pH7.0) and again fractions with high levels of pneumolysin derivative are pooled, concentrated by

ultrafiltration and stored in 50% glycerol at -15°C. The final product is greater than 95% pure, as judged by SDS-polyacrylamide gel electrophoresis. Hydrophobic interaction chromatography on Phenyl-Sepharose is an alternative purification which could also be used. However it is to be understood that this is only one method of purification of the altered pneumolysin, and other, alternative methods (including High Pressure Liquid Chromatography) may be employed.

This purified altered pneumolysin can then be administered as a vaccine at appropriate levels, either by itself or in combination with other antigens. In one form the pneumolysin may be conjugated with polysaccharide derived from any one or more of the variety of pneumococcal strains described above.

The mutant pneumolysin can be conjugated to the various serotypes of polysaccharide by a range of methods. The first involves preparation of an activated polysaccharide by treating pure polysaccharide (available commercially) with cyanogen-bromide and adipicacid dihydrzide (ADH). The ADH-polysaccharide is then combined with the mutant pneumolysin in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide - HCl. Conjugated material is separated from the reactants by chromatography through Sepharose CL-4B.

Alternatively, the polysaccharide-mutant pneumolysin conjugates can be prepared using bifunctional reagents such as N-succinimidyl-6(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH). Pure polysaccharide dissolved in phosphate buffered saline, is reacted with SANPAH in the presence of a strong white light source. Unreacted SANPAH is then separated from activated polysaccharide by chromatography on Sephadex G-50. Activated polysaccharide is then conjugated to the mutant pneumolysin in 0.2M borate buffer (pH8.5). Any excess reactive groups are then blocked with lysine, and the polysaccharide-protein conjugate is separated from the other reactants by chromatography on Sepharose CL-4B. Conjugates could also be prepared by reductive amination with cyanoborohydride.

Alternatively another protein, such as inactivated tetanus toxin, can be conjugated with the desired polysaccharides and altered pneumolysin can be added to the vaccine in an unconjugated form.

This then describes the best method of performing the invention however it is to be understood that the invention is not limited thereto.

1. An altered pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

2. An altered pneumolysin as in claim one having reduced complement binding activity as compared to wild-type pneumolysin.

3. An altered pneumolysin as in any one of claims 1 or 2 having reduced Fc binding activity as compared to wild-type pneumolysin.

4. An altered pneumolysin as in any one of claims 1, 2, or 3 wherein said altered pneumolysin is altered by reason of one or more amino acid substitutions within wild type pneumolysin.

5. An altered pneumolysin having the following amino acid sequence:-

Met	Ala	Asn	Lys	Ala	Val	Asn	Asp	Phe	Ile	Leu	Ala	Met
1										11		
Asn	Tyr	Asp	Lys	Lys	Lys	Leu	Leu	Thr	His	Gln	Gly	Glu
							21					
Ser	Ile	Glu	Asn	Arg	Phe	Ile	Lys	Glu	Gly	Asn	Gln	Leu
				31								
Pro	Asp	Glu	Phe	Val	Val	Ile	Glu	Arg	Lys	Lys	Arg	Ser
	41										51	
Leu	Ser	Thr	Asn	Thr	Ser	Asp	Ile	Ser	Val	Thr	Ala	Thr
								61				
Asn	Asp	Ser	Arg	Leu	Tyr	Pro	Gly	Ala	Leu	Leu	Val	Val
					71							
Asp	Glu	Thr	Leu	Leu	Glu	Asn	Asn	Pro	Thr	Leu	Leu	Ala
		81										91
Val	Asp	Arg	Ala	Pro	Met	Thr	Tyr	Ser	Ile	Asp	Leu	Pro
									101			
Gly	Leu	Ala	Ser	Ser	Asp	Ser	Phe	Leu	Gln	Val	Glu	Asp
						111						
Pro	Ser	Asn	Ser	Ser	Val	Arg	Gly	Ala	Val	Asn	Asp	Leu
			121									
Leu	Ala	Lys	Trp	His	Gln	Asp	Tyr	Gly	Gln	Val	Asn	Asn
131										141		
Val	Pro	Ala	Arg	Met	Gln	Tyr	Glu	Lys	Ile	Thr	Ala	His
							151					
Ser	Met	Glu	Gln	Leu	Lys	Val	Lys	Phe	Gly	Ser	Asp	Phe
				161								
Glu	Lys	Thr	Gly	Asn	Ser	Leu	Asp	Ile	Asp	Phe	Asn	Ser
	171										181	
Val	His	Ser	Gly	Glu	Lys	Gln	Ile	Gln	Ile	Val	Asn	Phe
								191				

Lys Gln Ile Tyr Tyr Thr Val Ser Val Asp Ala Val Lys
201

Asn Pro Gly Asp Val Phe Gln Asp Thr Val Thr Val Glu
211 221

Asp Leu Lys Gln Arg Gly Ile Ser Ala Glu Arg Pro Leu
231

Val Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr
241

Leu Lys Leu Glu Thr Thr Ser Lys Ser Asp Glu Val Glu
251

Ala Ala Phe Glu Ala Leu Ile Lys Gly Val Lys Val Ala
261 271

Pro Gln Thr Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu
281

Val Lys Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly
291

Ala Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp
301 311

Leu Ile Gln Glu Gly Ser Arg Phe Thr Ala Asp His Pro
321

Gly Leu Pro Ile Ser Tyr Thr Thr Ser Phe Leu Arg Asp
331

Asn Val Val Ala Thr Phe Gln Asn Ser Thr Asp Tyr Val
341 351

Glu Thr Lys Val Thr Ala Tyr Arg Asn Gly Asp Leu Leu
361

Leu Asp R_1 Ser Gly Ala Tyr Val Ala Gln Tyr Tyr Ile
371

Thr R_2 Asp Glu Leu Ser R_3 R_4 His Gln Gly Lys Glu
381

Val Leu Thr Pro Lys Ala R_5 Asp Arg Asn Gly Gln Asp
391 401

Leu Thr Ala His Phe Thr Thr Ser Ile Pro Leu Lys Gly
411

Asn Val Arg Asn Leu Ser Val Lys Ile Arg Glu R_6 Thr
421

Gly Leu Ala R_7 R_8 R_9 Trp Arg Thr Val Tyr Glu Lys
431 441

Thr Asp Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile
451

Trp Gly Thr Thr Leu Tyr Pro Gln Val Glu Asp Lys Val
461

Glu Asn Asp
471

wherein R_1 is His or Arg, R_2 is Trp or Phe, R_3 is Tyr or Phe, R_4 is Asp or Asn, R_5 is Trp or Phe, R_6 is Cys, Gly, or Ser, R_7 is Trp or Phe, R_8 is Glu, or Asp, R_9 is Trp or Phe, and wherein at least one of the residues R_1, R_6, R_7, R_8, or R_9 is other than wild-type.

6. An altered pneumolysin as in claim 5 wherein wherein R_1 is Arg, R_2 is Trp, R_3 is Tyr, R_4 is Asn, R_5 is Trp, R_6 is Cys, R_7 is Trp, R_8 is Glu, and R_9 is Trp.

7. A vaccine including an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal being reactive to wild-type pneumolysin.

8. A vaccine as in claim 7 wherein the altered pneumolysin is as claimed in any one of claims 2 to 6.

9. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier and non-conjugated protein material, the capsular polysaccharide material being derived from any one or more than one of the *Streptococcus pneumoniae* serotypes, and the non-conjugated protein material being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

10. A vaccine as in claim 9 wherein the capsular material is derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A, 6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

11. A vaccine as in either claim 9 or 10 wherein the altered pneumolysin is as claimed in as in any one of claims 2 to 6.

12. A vaccine comprising capsular polysaccharide material conjugated with a protein carrier, the capsular polysaccharide material being derived from any one or more than one of the *Streptococcus pneumoniae* serotypes, and the protein carrier being an altered pneumolysin, said altered pneumolysin being non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

13. A vaccine as in claim 12 wherein the capsular material is derived from any one or more of the *Streptococcus pneumoniae* serotypes 6A,

6B, 14, 18C, 19A, 19F, 23F, 1, 2, 3, 4, 5, 7F, 8, 9N, 9V, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

14. A vaccine as in either claim 12 or 13 wherein the altered pneumolysin is as claimed in any one of claims 2 to 6.

15. A recombinant plasmid including a DNA sequence encoding an altered pneumolysin as claimed in any one of claims 1 to 6.

16. A hybrid host cell including a recombinant plasmid as claimed in claim 9 said recombinant plasmid including an inducible expression control operable for expression of said altered pneumolysin encoding DNA within a host cell.

17. A method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from an expression system including a recombinant plasmid with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal reactive to wild type pneumolysin.

18. A method of producing an altered pneumolysin including the steps of purifying said altered pneumolysin from a culture of a host cell including a recombinant clone with DNA encoding an altered pneumolysin said pneumolysin being substantially non-toxic and being capable of eliciting an immune response in an animal said immune response being reactive to wild type pneumolysin.

19. A method of producing a vaccine including the step of amplifying a recombinant clone encoding an altered pneumolysin, inducing transcription and translation of said cloned material, the purification of altered pneumolysin, and the step of conjugating the altered pneumolysin with a capsular polysaccharide, the altered pneumolysin having substantially reduced toxic activity as compared with wild type pneumolysin.

20 A method of producing a vaccine as in claim 19 wherein said altered pneumolysin is as claimed in any one of claims 2 to 6.

21. An altered pneumolysin as hereinbefore described with reference to the examples.

22. A vaccine including an altered pneumolysin as hereinbefore described with reference to the examples.

23. A method of producing a vaccine as hereinbefore described with reference to the examples.

AGATGGCAAA TAAAGCAGTA AATGACTTTA TACTAGCTAT GAATTACGAT
AAAAAGAAAC TCTTGACCCA TCAGGGAGAA AGTATTGAAA ATCGTTTCAT
CAAAGAGGGT AATCAGCTAC CCGATGAGTT TGTTGTTATC GAAAGAAAGA
AGCGGAGCTT GTCGACAAAT ACAAGTGATA TTTCTGTAAC AGCTACCAAC
GACAGTCGCC TCTATCCTGG AGCACTTCTC GTAGTGGATG AGACCTTGTT
AGAGAATAAT CCCACTCTTC TTGCGGTTGA TCGTGCTCCG ATGACTTATA
GTATTGATTT GCCTGGTTTG GCAAGTAGCG ATAGCTTTCT CCAAGTGGAA
GACCCCAGCA ATTCAAGTGT TCGCGGAGCG GTAAACGATT TGTTGGCTAA
GTGGCATCAA GATTATGGTC AGGTCAATAA TGTCCCAGCT AGAATGCAGT
ATGAAAAAAT AACGGCTCAC AGCATGGAAC AACTCAAGGT CAAGTTTGGT
TCTGACTTTG AAAAGACAGG GAATTCTCTT GATATTGATT TTAACTCTGT
CCATTCAGGT GAAAAGCAGA TTCAGATTGT TAATTTTAAG CAGATTTATT
ATACAGTCAG CGTAGACGCT GTTAAAAATC CAGGAGATGT GTTTCAAGAT
ACTGTAACGG TAGAGGATTT AAAACAGAGA GGAATTTCTG CAGAGCGTCC
TTTGGTCTAT ATTTCGAGTG TTGCTTATGG GCGCCAAGTC TATCTCAAGT
TGGAAACCAC GAGTAAGAGT GATGAAGTAG AGGCTGCTTT TGAAGCTTTG
ATAAAAGGAG TCAAGGTAGC TCCTCAGACA GAGTGGAAGC AGATTTTGGA
CAATACAGAA GTGAAGGCGG TTATTTTAGG GGGCGACCCA AGTTCGGGTG
CCCGAGTTGT AACAGGCAAG GTGGATATGG TAGAGGACTT GATTCAAGAA
GGCAGTCGCT TTACAGCAGA TCATCCAGGC TTGCCGATTT CCTATACAAC
TTCTTTTTTA CGTGACAATG TAGTTGCGAC CTTTCAAAAC AGTACAGACT
ATGTTGAGAC TAAGGTTACA GCTTACAGAA ACGGAGATTT ACTGCTGGAT
CATAGTGGTG CCTATGTTGC CCAATATTAT ATTACTTGGG ATGAATTATC
CTATGATCAT CAAGGTAAGG AAGTCTTGAC TCCTAAGGCT TGGGACAGAA
ATGGGCAGGA TTTGACGGCT CACTTTACCA CTAGTATTCC TTTAAAAGGG
AATGTTCGTA ATCTCTCTGT CAAAATTAGA GAGTGTACCG GGCTTGCCTG
GGAATGGTGG CGTACGGTTT ATGAAAAAAC CGATTTGCCA CTAGTGCGTA
AGCGGACGAT TTCTATTTGG GGAACAACTC TCTATCCTCA GGTAGAGGAT
AAGGTAGAAA ATGAC

FIGURE 1 DNA sequence of pneumolysin gene. ATG start codon underlined

CCATGGCAAA TAAAGCAGTA AATGACTTTA TACTAGCTAT GAATTACGAT
AAAAAGAAAC TCTTGACCCA TCAGGGAGAA AGTATTGAAA ATCGTTTCAT
CAAAGAGGGT AATCAGCTAC CCGATGAGTT TGTTGTTATC GAAAGAAAGA
AGCGGAGCTT GTCGACAAAT ACAAGTGATA TTTCTGTAAC AGCTACCAAC
GACAGTCGCC TCTATCCTGG AGCACTTCTC GTAGTGGATG AGACCTTGTT
AGAGAATAAT CCCACTCTTC TTGCGGTTGA TCGTGCTCCG ATGACTTATA
GTATTGATTT GCCTGGTTTG GCAAGTAGCG ATAGCTTTCT CCAAGTGGAA
GACCCCAGCA ATTCAAGTGT TCGCGGAGCG GTAAACGATT TGTTGGCTAA
GTGGCATCAA GATTATGGTC AGGTCAATAA TGTCCCAGCT AGAATGCAGT
ATGAAAAAAT AACGGCTCAC AGCATGGAAC AACTCAAGGT CAAGTTTGGT
TCTGACTTTG AAAAGACAGG GAATTCTCTT GATATTGATT TTAACTCTGT
CCATTCAGGT GAAAAGCAGA TTCAGATTGT TAATTTTAAG CAGATTTATT
ATACAGTCAG CGTAGACGCT GTTAAAAATC CAGGAGATGT GTTTCAAGAT
ACTGTAACGG TAGAGGATTT AAAACAGAGA GGAATTTCTG CAGAGCGTCC
TTTGGTCTAT ATTTCGAGTG TTGCTTATGG GCGCCAAGTC TATCTCAAGT
TGGAAACCAC GAGTAAGAGT GATGAAGTAG AGGCTGCTTT TGAAGCTTTG
ATAAAAGGAG TCAAGGTAGC TCCTCAGACA GAGTGGAAGC AGATTTTGGA
CAATACAGAA GTGAAGGCGG TTATTTTAGG GGGCGACCCA AGTTCGGGTG
CCCGAGTTGT AACAGGCAAG GTGGATATGG TAGAGGACTT GATTCAAGAA
GGCAGTCGCT TTACAGCAGA TCATCCAGGC TTGCCGATTT CCTATACAAC
TTCTTTTTTA CGTGACAATG TAGTTGCGAC CTTTCAAAAC AGTACAGACT
ATGTTGAGAC TAAGGTTACA GCTTACAGAA ACGGAGATTT ACTGCTGGAT
CATAGTGGTG CCTATGTTGC CCAATATTAT ATTACTTGGG ATGAATTATC
CTATGATCAT CAAGGTAAGG AAGTCTTGAC TCCTAAGGCT TGGGACAGAA
ATGGGCAGGA TTTGACGGCT CACTTTACCA CTAGTATTCC TTTAAAAGGG
AATGTTCGTA ATCTCTCTGT CAAAATTAGA GAGTGTACCG GGCTTGCCTG
GGAATGGTGG CGTACGGTTT ATGAAAAAAC CGATTTGCCA CTAGTGCGTA
AGCGGACGAT TTCTATTTGG GGAACAACTC TCTATCCTCA GGTAGAGGAT
AAGGTAGAAA ATGAC

FIGURE 2 DNA sequence of modified pneumolysin gene. An NcoI restriction site (underlined) has been introduced at the start codon

Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala Met
1 11

Asn Tyr Asp Lys Lys Lys Leu Leu Thr His Gln Gly Glu
21

Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly Asn Gln Leu
31

Pro Asp Glu Phe Val Val Ile Glu Arg Lys Lys Arg Ser
41 51

Leu Ser Thr Asn Thr Ser Asp Ile Ser Val Thr Ala Thr
61

Asn Asp Ser Arg Leu Tyr Pro Gly Ala Leu Leu Val Val
71

Asp Glu Thr Leu Leu Glu Asn Asn Pro Thr Leu Leu Ala
81 91

Val Asp Arg Ala Pro Met Thr Tyr Ser Ile Asp Leu Pro
101

Gly Leu Ala Ser Ser Asp Ser Phe Leu Gln Val Glu Asp
111

Pro Ser Asn Ser Ser Val Arg Gly Ala Val Asn Asp Leu
121

Leu Ala Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn
131 141

Val Pro Ala Arg Met Gln Tyr Glu Lys Ile Thr Ala His
151

Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp Phe
161

Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe Asn Ser
171 181

Val His Ser Gly Glu Lys Gln Ile Gln Ile Val Asn Phe
191

Lys Gln Ile Tyr Tyr Thr Val Ser Val Asp Ala Val Lys
201

Asn Pro Gly Asp Val Phe Gln Asp Thr Val Thr Val Glu
211 221

Asp Leu Lys Gln Arg Gly Ile Ser Ala Glu Arg Pro Leu
231

Val Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr
241

Leu Lys Leu Glu Thr Thr Ser Lys Ser Asp Glu Val Glu
251

Ala Ala Phe Glu Ala Leu Ile Lys Gly Val Lys Val Ala
261 271

Pro Gln Thr Glu Trp Lys Gln Ile Leu Asp Asn Thr Glu
281

Val Lys Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly
291

Ala Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp
301 311

Leu Ile Gln Glu Gly Ser Arg Phe Thr Ala Asp His Pro
321

Gly Leu Pro Ile Ser Tyr Thr Thr Ser Phe Leu Arg Asp
331

Asn Val Val Ala Thr Phe Gln Asn Ser Thr Asp Tyr Val
341 351

Glu Thr Lys Val Thr Ala Tyr Arg Asn Gly Asp Leu Leu
361

Leu Asp His Ser Gly Ala Tyr Val Ala Gln Tyr Tyr Ile
371

Thr Trp Asp Glu Leu Ser Tyr Asp His Gln Gly Lys Glu
381

Val Leu Thr Pro Lys Ala Trp Asp Arg Asn Gly Gln Asp
391 401

Leu Thr Ala His Phe Thr Thr Ser Ile Pro Leu Lys Gly
411

Asn Val Arg Asn Leu Ser Val Lys Ile Arg Glu Cys Thr
421

Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Tyr Glu Lys
431 441

Thr Asp Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile
451

Trp Gly Thr Thr Leu Tyr Pro Gln Val Glu Asp Lys Val
461

Glu Asn Asp
471

Figure 3

Met Ala Asn Lys Ala Val Asn Asp Phe Ile Leu Ala Met
1 11

Asn Tyr Asp Lys Lys Lys Leu Leu Thr His Gln Gly Glu
21

Ser Ile Glu Asn Arg Phe Ile Lys Glu Gly Asn Gln Leu
31

Pro Asp Glu Phe Val Val Ile Glu Arg Lys Lys Arg Ser
41 51

Leu Ser Thr Asn Thr Ser Asp Ile Ser Val Thr Ala Thr
61

Asn Asp Ser Arg Leu Tyr Pro Gly Ala Leu Leu Val Val
71

Asp Glu Thr Leu Leu Glu Asn Asn Pro Thr Leu Leu Ala
81 91

Val Asp Arg Ala Pro Met Thr Tyr Ser Ile Asp Leu Pro
101

Gly Leu Ala Ser Ser Asp Ser Phe Leu Gln Val Glu Asp
111

Pro Ser Asn Ser Ser Val Arg Gly Ala Val Asn Asp Leu
121

Leu Ala Lys Trp His Gln Asp Tyr Gly Gln Val Asn Asn
131 141

Val Pro Ala Arg Met Gln Tyr Glu Lys Ile Thr Ala His
151

Ser Met Glu Gln Leu Lys Val Lys Phe Gly Ser Asp Phe
161

Glu Lys Thr Gly Asn Ser Leu Asp Ile Asp Phe Asn Ser
171 181

Val His Ser Gly Glu Lys Gln Ile Gln Ile Val Asn Phe
191

Lys Gln Ile Tyr Tyr Thr Val Ser Val Asp Ala Val Lys
201

Asn Pro Gly Asp Val Phe Gln Asp Thr Val Thr Val Glu
211 221

Asp Leu Lys Gln Arg Gly Ile Ser Ala Glu Arg Pro Leu
231

```
Val Tyr Ile  Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr
                         241

Leu Lys Leu Glu Thr Thr Ser Lys Ser Asp Glu Val Glu
            251
        Trp
         |
Ala Ala Phe Glu Ala Leu Ile  Lys Gly Val Lys Val Ala
261                                      271
                Phe
                 |
Pro Gln Thr Glu Trp Lys Gln Ile  Leu Asp Asn Thr Glu
                         281

Val Lys Ala Val Ile Leu Gly Gly Asp Pro Ser Ser Gly
                    291

Ala Arg Val Val Thr Gly Lys Val Asp Met Val Glu Asp
    301                                      311

Leu Ile  Gln Glu Gly Ser Arg Phe Thr Ala Asp His Pro
                         321

Gly Leu Pro Ile  Ser Tyr Thr Thr Ser Phe Leu Arg Asp
                    331

Asn Val Val Ala Thr Phe Gln Asn Ser Thr Asp Tyr Val
        341                                      351

Glu Thr Lys Val Thr Ala Tyr Arg Asn Gly Asp Leu Leu
                              361
         Arg
          |
Leu Asp His Ser Gly Ala Tyr Val Ala Gln Tyr Tyr Ile
                    371

    Phe                  Phe Asn
     |                    |   |
Thr Trp Asp Glu Leu Ser Tyr Asp His Gln Gly Lys Glu
            381
                         Phe
                          |
Val Leu Thr Pro Lys Ala Trp Asp Arg Asn Gly Gln Asp
391                                      401

Leu Thr Ala His Phe Thr Thr Ser Ile  Pro Leu Lys Gly
                         411
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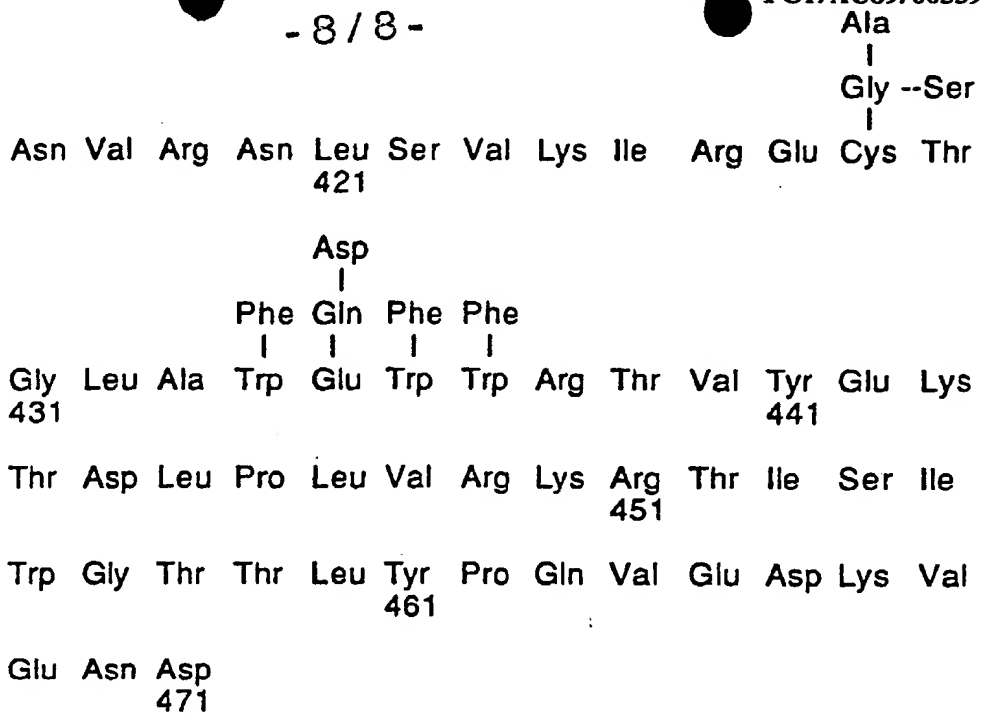
Ala
Gly --Ser
Asn Val Arg Asn Leu Ser Val Lys Ile Arg Glu Cys Thr
421
Asp
Phe Gln Phe Phe
Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Tyr Glu Lys
431 441
Thr Asp Leu Pro Leu Val Arg Lys Arg Thr Ile Ser Ile
451
Trp Gly Thr Thr Leu Tyr Pro Gln Val Glu Asp Lys Val
461
Glu Asn Asp
471

Figure 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00539

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.[5] C07K 13/00, C12P 21/00, C12N 15/31, C07H 21/04

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	Derwent databases: WPI, WPIL, USPA: keywords STREPTOCOCCUS PNEUMONIAE, PNEUMOCOCCUS, PNEUMOLYSIN HAEMOLYSIN, HEMOLYSIN, CBR, COMPLEMENT BINDING REGION CPR, REACTIVE PROTEIN

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Aust Class: C07K 13/00, 15/04
C12N 15/31 CHEM ABS using keywords above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
PX	Infection and Immunity, Vol 57 (8) Aug 1989 p2547-2552 F.K. SAUNDERS et al "Pneumolysin, the Thiol-Activated Toxin of Streptococcus pneumoniae, does not require a Thiol Group for In Vitro Activity"	1, 4-5, 15-21
A	Infection and Immunity, Vol 55 (5) May 1987, p1184-1189 WALKER, J.A. et al "Molecular Cloning, Characterization, and complete Nucleotide Sequence of the Gene for Pneumolysin, the Sulfhydryl-Activated Toxin of Streptococcus pneumoniae	1-22
A	Journal of Clinical Microbiology Feb 1987 p222-225 Krzysztof Kanclerski et al "Production and Purification of Streptococcus pneumoniae Hemolysin (Pneumolysin)	1-22

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6 April 1990 (06.04.90)	12 April 1990
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	R. SAWYER

Form PCT/ISA/210 (second sheet) (January 1985)